Role of Pattern Recognition Receptors in the Modulation of Antimicrobial Peptide Expression in the Corneal Epithelial Innate Response to *F. solani*

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Purpose. Fusarium solani (*F. solani*) keratitis is a potentially sight-threatening fungal infection of the cornea. Antimicrobial peptides (AMPs), such as human β-defensins (hBDs) and cathelicidins, essential components of the immune system, likely have a protective role against *F. solani* keratitis. We examined the role of pattern recognition receptors (PRRs), Dectin-1, and TLR2 in *F. solani*–induced modulation of AMP expression in vitro.

Methods. Human corneal epithelial cells (HCECs) were exposed to heat-inactivated *F. solani* or pathogen-associated molecular patterns (PAMPs) of *F. solani* (Zymosan or Zymosan Depleted) for 6, 12, or 24 hours following which AMP mRNA and protein levels were determined. Involvement of TLR2 and Dectin-1 was confirmed by using siRNA knock-down (TLR2 and Dectin-1) or chemical inhibitor BAY 61-3606 (Dectin-1). The functional significance of AMP upregulation was tested using culture supernatant from *F. solani* or PAMP-treated HCECs against *F. solani* in the presence of hBD2 or LL37 neutralizing antibody.

Results. We confirm that HCECs express Dectin-1 and TLR2. HCECs demonstrated upregulation of AMPs hBD2 and cathelicidin LL37 following exposure to heat-inactivated *F. solani* or PAMPs. TLR2 and Dectin-1 knockdown and BAY 61-3606 treatment decreased AMP mRNA upregulation confirming PRR involvement. The culture supernatant from *F. solani* or PAMP-treated HCECs showed substantial killing of *F. solani* and hBD2 or LL37 neutralizing antibody significantly decreased this effect implicating involvement of these AMPs.

Conclusions. These findings demonstrate that Dectin-1 and TLR2 have an important role in regulating *F. solani*-induced AMP expression in corneal epithelial cells.

Keywords: *F. solani*, keratitis, defensins, cathelicidins, pattern recognition receptors

Fungal keratitis (keratomycosis) is a potentially devastating ocular infection that may result in permanent visual impairment and even blindness. It is endemic in tropical regions, such as parts of Central and South America, Africa, Asia, and in the southeastern and central United States.[1,2] Fungal keratitis is linked historically to ocular trauma resulting from vegetative matter contaminated with soil as reported with most cases from developing countries, such as India and Ghana.[3,4] However, contact lens wear is recognized as the major predisposing factor for fungal keratitis in industrialized nations. For example, in the United States, contact lens wear accounts for 37% and ocular trauma for 25% of fungal keratitis cases.[1] While a range of fungi have been identified as the culprit, *Fusarium* species are the most commonly isolated organisms in fungal keratitis. *F. solani* in particular has been implicated as the causative pathogen in more than 30% of cases[2,5–7] and was identified as the culprit in the 2004 to 2006 worldwide epidemic associated with contact lens wear.[8,9] Despite *F. solani* keratitis being a critical cause of vision loss and blindness in developing and developed countries, only a few published studies have addressed the underlying pathology and host defense mechanisms to protect and limit the damage to the cornea.

Antimicrobial peptides (AMPs) form an integral part of the innate immune system and provide defense against a range of pathogens as well as modulating immune responses and wound healing.[10] At the human ocular surface the primary AMPs produced by the epithelial cells are β-defensins (hBDs) and the cathelicidin LL37.[11] These help provide a baseline defense against invading pathogens and several are upregulated in response to infection and inflammatory stimuli.[12–14] We have shown previously that there is increased expression of β-defensins and cathelicidin in a murine model of *F. solani* keratitis and that severity of infection is increased in AMP knockout or knockdown mice, thus implying an important role for AMPs in defense against *F. solani* infection.[15]

To better understand the mechanism underlying *F. solani* stimulation of corneal AMP expression, our studies focused on two pattern recognition receptors (PRRs), Toll-like receptor 2 (TLR2) and Dectin-1, as likely primary mediators of enhanced AMP expression. TLR2 is one of several TLRs expressed by the ocular surface epithelia[16] and can interact with a number of fungal-associated pathogen molecular patterns (PAMPs) such as β-glucans and phospholipomannans.[17] TLR2 activation is known to increase human corneal epithelial (HCEC) AMP expression[18] and in part mediate proinflammatory cytokine secretion induced by HCEC exposure to inactive hyphal fragments of *F. solani*.[19] TLR2 also is one of several TLRs showing enhanced expression in humans corneas infected by *F. solani*.[20] Dectin-1 belongs to the C-type lectin-like receptor

family, which are the primary PRR group for the recognition of fungi.[21] It recognizes β-1,3-glucans from the cell wall of many fungi and, like some other C-type lectins, signals NF-kB activation through the syk/CARD9 pathway.[22] Dectin-1 is expressed by HCEC[23,24] and it is upregulated in *Fusarium*-infected human corneas.[25] Little is known about the role of Dectin-1 in *F. solani* keratitis although it has been reported to mediate increased HCEC IL-8 expression in response to *Aspergillus fumigatus*[26] and is implicated as the major PRR on resident corneal macrophages and dendritic cells that triggers the immune response in a murine model of *Aspergillus* keratitis.[27] We investigated a novel role for Dectin-1 in mediating *F. solani*-stimulated AMP expression by HCEC as well as collaboration between Dectin-1 and TLR2 as has been shown in other antifungal responses.[22]

Materials and Methods

Fungi

F. solani (strain 36031; American Type Culture Collection, Manassas, VA, USA), a pathogenic strain obtained from a patient with *F. solani* keratitis that is capable of producing murine keratomycosis,[15] was cultured in Sabouraud dextrose (SD) agar (Difco, Detroit, MI, USA) for 3 days at 30°C. A colony of *F. solani* was inoculated into 4 mL of SD broth and grown aerobically overnight with shaking (250 rpm) at 30°C. Then, 250 μL of fungal suspension were inoculated into 50 mL of fresh SD broth at 30°C, 250 rpm for 48 hours to expand the culture. The turbidity of this hyphal suspension was adjusted to an optical density (OD) of 1 at 600 nm which corresponds to approximately 5×10^5culturable units (CU). The hyphal suspension then was diluted by centrifuging at 300*g* for 5 minutes, and resuspending in media to yield 1×10^5 CU/ mL. Aliquots (100 μL) were heat-inactivated by incubating the suspension at 100°C for 20 minutes. The aliquots were stored at −80°C until used in an experiment.

Human Corneal Epithelial Cells

Telomerase modified human corneal epithelial cells (hTCEpi)[28] were grown at 37°C with 5% CO_2 in KG-2 keratinocyte growth medium-2 (Lonza, Houston, TX, USA) in the presence of growth factors and 50 μg/mL Normocin (InvivoGen, San Diego, CA, USA). Findings from some experiments were confirmed in primary cultured HCEC (passages 1 and 2) prepared from pairs of normal cadaveric corneas received within 2 to 5 days of death based on a method previously described.[12] Cells were plated in 6-well plates and treated with heat-inactivated *F. solani* (prepared as described above) or PAMPs Zymosan, Zymosan Depleted (InvivoGen) or the combination of the two at 10 μg/mL for 6, 12, or 24 hours. In PRR knockdown experiments 10 nM nonspecific or 3 different specific TLR2 (#1-s168, #2-s169, and #3-s170) or Dectin-1 siRNAs (#1-s34783, #2-s34785, and #3-s227204; Ambion, Austin, TX, USA) were tested. The siRNA, which demonstrated the highest percent knockdown, was reconfirmed using RT-PCR and then used in subsequent experiments. Briefly, hTCEpi cells were treated with HiPerFect transfection reagent (Qiagen, Valencia, CA, USA) as vehicle, 10 nM nonspecific or TLR2 (#3-s170), or Dectin-1 siRNAs (#3-s227204) for 48 hours and heat-inactivated *F. solani* for the final 6 or 24 hours, following which cells were processed for mRNA or protein quantitation. In some experiments cells were treated for 48 hours with 10 μM BAY 61-3601 (Emd Biosciences, Inc., San Diego, CA, USA) to block Syk, a known mediator of Dectin-1 activity.[29,30] Culture supernatants were harvested for protein analysis by immunoblot or to test for antifungal activity. Cell lysates were collected to determine mRNA levels for the AMPs hBD-1, -2, -3, and LL37.

RT-Polymerase Chain Reaction

Relative-quantitative real-time PCR was used to study AMP expression. Total RNA from primary HCECs and hTCEpi cells was extracted using an RNeasy Mini Kit (Qiagen) and quantified using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). RNA elution columns were DNAse (Qiagen) treated before RNA elution to avoid genomic DNA contamination. Two μg of total RNA were reverse transcribed to cDNA using SuperScript III First-Strand Synthesis System (Invitrogen, Carlsbad, CA, USA). RNAse free-water in place of RNA served as a no-template control and reactions without reverse transcriptase served as a no-RT control. Relative-quantitative real-time PCR amplification was performed using SYBR Green QPCR Mastermix kits (Stratagene, Santa Clara, CA, USA) with specific primer sequences as described previously,[13,18] at optimized concentrations. Primer sequences used for RPL-27, Dectin-1, and TLR2 were as follows: RPL-27 Forward: ATCGCCAAGAGACAAAGATAA, RPL-27 Reverse: TCTGAAGACATCCTTATTGAC; Dectin-1 Forward: ACTCTCAAAGCAATACCAGGA, Dectin-1 Reverse: CGCCAAG GAGGAGATGCA; TRL2 Forward: CCTGGCCCTCTCTACAAA CTT, TLR2 Reverse: ACTGTGTATTCGTGTGCTGGATA. The PCR reaction included an initial 10-minute denaturation at 95°C, then amplification was performed for 40 cycles: denaturation, 95°C for 30 seconds; annealing, 56°C for 1 minute; and extension, 72°C for 30 seconds. Data analysis was performed using the Stratagene Mx3005p software and disassociation melt curves were analyzed to ensure reaction specificity. Amplified gene products were normalized to RPL27, the internal control, and calibrated to the untreated samples. The control samples were normalized to one and the relative change of treated versus control samples was determined. For each experiment, the samples were analyzed in triplicate and the mean relative quantity of AMP expression was calculated.

One step RT-PCR was used to investigate expression and knockdown of Dectin-1 and TLR2. Total RNA from the samples was extracted as described previously.[18] RT-PCR was performed using a Superscript One-step RT-PCR kit (Qiagen) with 40 cycles of amplification: denaturation, 94°C for 1 minute; annealing, 58°C for 1 minute; extension at 72°C for 2 minutes. RT-PCR products were separated on 1.8% agarose gels and were visualized using an Alpha Imager Gel documentation system (Alpha Innotec, San Leandro, CA, USA).

Immunoblotting and Flow Cytometry

AMPs hBD2 and LL37 were detected in cell culture supernatants by immunoblot as described previously.[18] hTCEpi cells were treated with heat-inactivated *F. solani* or PAMPs for 24 hours and culture supernatants were collected, centrifuged at 1400*g* for 2 minutes to remove cell debris, and the samples were stored at −80°C until analysis. Samples were blotted onto PVDF membranes using a microfiltration apparatus (Biodot, Irvine, CA, USA) then the membrane was incubated for 1 hour at room temperature with Tris-buffered saline (TBS) containing 5% nonfat powdered milk to block the nonspecific binding sites. The membrane then was incubated with rabbit anti-human hBD2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or LL37 (donated by R. I. Lehrer) diluted 1 to 1000 in TBS containing 5% nonfat powdered milk, 5% goat serum, and 0.1% Tween 20 overnight at 4°C. The membrane then was washed (6×5 minutes; TBST) and incubated for 45 minutes at room temperature with a horseradish peroxidase–conjugated goat

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anti-rabbit antibody (Jackson Laboratories, West Grove, PA, USA), diluted 1:50,000 for LL37 and 1:10,000 for hBD2 in TBS containing 5% nonfat powdered milk.

Western blotting was performed to detect Dectin-1 or TLR2 and expression of Syk and phosphorylated-Syk in cell lysates. Cells were lysed using radioimmunoprecipitation assay buffer (RIPA buffer) for Dectin-1 and TLR2 immunoblotting and NP40 cell lysis buffer for the Syk and p-Syk assay. Protein quantitation was performed using a BCA assay kit (Fischer, Windsor, CT, USA). Equal amounts of protein (30 μg for Dectin-1 and TLR2 or 20 μg for Syk and p-Syk) were mixed with loading buffer and separated on 4% to 20% precast-polyacrylamide gels (Bio-Rad Laboratories, Hercules, CA, USA). The proteins then were transferred onto 0.4 μm nitrocellulose membranes (Bio-Rad Laboratories), which were blocked with 5% milk for Dectin-1 or TLR2 and 5% BSA for Syk proteins in 1 × TBST for 1 hour. The membranes then were incubated with goat anti-human Dectin-1/CLC7A antibody (R&D Systems, Minneapolis, MN, USA) at 1:1000 or mouse anti-human TLR2 antibody (Imgenex, San Diego, CA, USA) at 1:250 diluted in 1% milk in TBS with 0.1% Tween overnight at 4°C. The membranes were washed and incubated for 1 hour at room temperature with rabbit anti-goat HRP (Santa Cruz Biotechnology) or goat anti-mouse HRP at 1:4000 (Santa Cruz Biotechnology). For total Syk and p-Syk, the membranes were incubated in rabbit monoclonal primary antibodies (Cell Signaling, Davers, MA, USA) at 1 in 1000 dilution at 4°C for 48 hours. The membranes then were washed and incubated for 45 minutes at room temperature with goat anti-rabbit HRP at 1 in 8000 for p-Syk and 1 in 10,000 for total Syk (Pierce, Waltham, MA, USA). The membranes were stripped after imaging and blotted for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a loading control using mouse anti-GAPDH primary at 1 in 20,000 (Millipore, Kankakee, IL) and goat anti-mouse HRP (Santa Cruz Biotechnology) secondary antibodies.

Immunoreactivity was visualized by enhanced chemiluminescence ECL Prime Western Blot detection kits (GE Healthcare, Piscataway, NJ, USA). Images were captured using an Alpha Imager documentation system (Alpha Innotec). Densitometry was performed and data analyzed with Alpha Ease FC software. The densitometry data were expressed as relative pixel intensity of control, which was normalized to 1.

For flow cytometry hTCEpi cells treated with or without *F. solani*, TLR2 siRNA, or nonspecific siRNA were harvested and centrifuged at 400*g* for 5 minutes to generate a cell pellet. Cells (800,000) were blocked with 10% human serum (Sigma-Aldrich Corp., St. Louis, MO, USA) for 15 minutes, permeabilized, and fixed for 20 minutes using Cytofix/Cytoperm (BD Pharmingen, San Diego, CA, USA). Cells were washed and incubated with 2 μg/100 μL cell suspension mouse anti-human TLR2 antibody (Novus, Littleton, CO, USA) or mouse IgG2a as isotype control for 1 hour at 4°C. The cells then were incubated with anti-mouse IgG Alexa Fluor 488 (Molecular Probes, Eugene, OR, USA) at 1:300 for 30 minutes at 4°C. The samples were evaluated using a FACS Canto II (BD Biosciences, San Jose, CA, USA) and the data were analyzed using BD FacsDiva software (BD Biosciences).

Antifungal Assay

The antimicrobial activity of antimicrobial agent free culture supernatant harvested from hTCEpi cells treated with heat-inactivated *F. solani,* Zymosan, or Zymosan Depleted was tested against *F. solani* based on a previously described protocol.[18] Briefly, after 24 hours of treatment the culture media were collected, then centrifuged at 1400*g* for 2 minutes. One mL of the supernatant was incubated with freshly prepared *F. solani* at 30°C with shaking at 250 rpm for 4 hours; the culture media from untreated cells was used as control. At the end of the incubation, 10-fold serial dilutions were plated on SD agar plates and digital images were captured using an Alpha Imager documentation system and the number of CU counted after 48 hours of incubation.

To investigate the involvement of specific AMPs in the antifungal activity that was detected additional experiments were performed in which culture media from hTCEpi cells treated with heat-inactivated *F. solani*, Zymosan, or Zymosan Depleted was first incubated with antibody against LL37 (1 in 200, donated by R. Lehrer) or hBD2 (25 μg/mL; PeproTech, Rocky Hill, NJ, USA) or normal rabbit serum (control for LL37; Sigma-Aldrich Corp.) or goat IgG (control for hBD2; Santa Cruz Biotechnology) overnight at 4°C. The culture media then were incubated with *F. solani,* plated, and CU counted after 48 hours as described above.

Statistical Analysis

Multiple comparisons were made using ANOVA in conjunction with a Tukey's honestly significant difference (HSD) test to report mean differences. All experiments presenting quantitative data were repeated at least three times, except where stated, to ensure reproducibility. A P value <0.05 was considered statistically significant.

Results

AMP Expression Following *F. solani* Challenge in Human Corneal Epithelial Cells

Figure 1A shows the relative fold change in hBD-1, -2, -3, and LL37 mRNA expression at 6, 12, and 24 hours after challenge with heat-inactivated *F. solani* in hTCEpi cells. hBD-2, -3, and LL37 expression was upregulated at all time points after challenge compared to control. Peak increases were at 6 hours after challenge with the levels of mRNA expression decreasing thereafter. AMPs hBD2 and LL37 showed statistically significant upregulation by 107.02 ± 3.3-fold and 77.24 ± 2.2-fold respectively, compared to untreated control ($P < 0.0001$) after 6 hours of *F. solani* treatment. Although increased, expression of hBD3 at all time points and that of hBD2 and LL37 at 12 and 24 hours was not statistically significantly upregulated. Expression of hBD1 was not changed at any time point. Protein expression of hBD2 and LL37 was examined in cell culture supernatant by immunoblotting. As shown in Figure 1B there was an increase in the secretion of hBD2 and LL37 protein in *F. solani*-treated cells, findings in keeping with the mRNA analysis. *Candida albicans*–treated hTCEpi cells were used as a positive control as previous studies have demonstrated AMP modulation by this fungus.[31] Figure 1C shows normalized densitometry findings from the immunoblots demonstrating an increase in hBD2 protein by 18.33 ± 4.3-fold and 3.56 ± 1.75-fold and LL37 protein by 4.01 ± 0.8-fold and 3.03 ± 0.5-fold in *F. solani*– and *C. albicans*–treated cells compared to untreated control, respectively. Upregulation of hBD2 and LL37 expression was confirmed in primary cultured HCECs following *F. solani* challenge as shown in Figure 2F.

Time-Dependent Expression of AMPs in PAMP-Treated Human Corneal Epithelial Cells

Figure 2 shows hTCEpi cell upregulation of AMPs following treatment with fungal PAMPs, Zymosan (an agonist that acts directly and preferentially on TLR2, although is known to stimulate TLR2 and Dectin-1[32,33]), Zymosan Depleted (Dectin-1 agonist only), or the combination of the two. Initial experi-

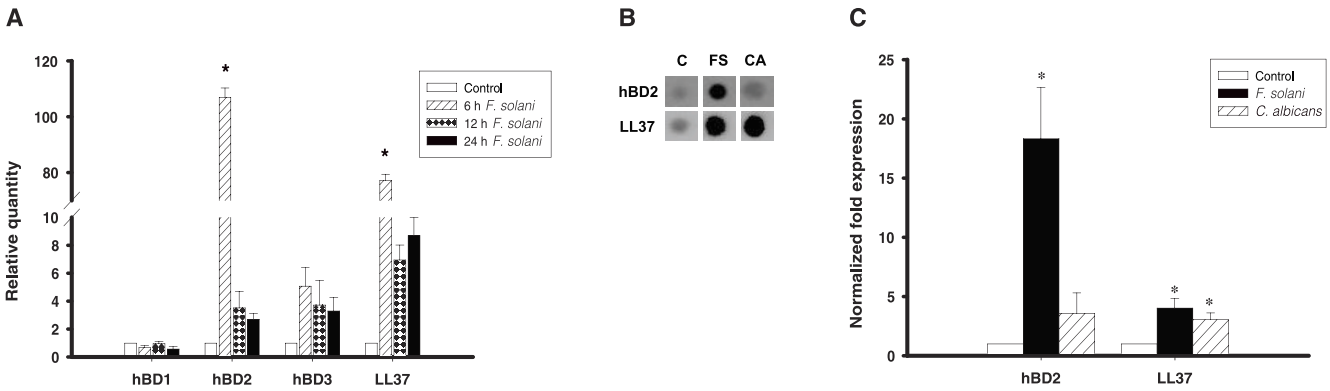

Figure 1. AMP expression in *F. solani* treated hTCEpi. (**A**) AMP expression determined 6, 12, and 24 hours after treatment with *F. solani* indicated significant upregulation of hBD2 and LL37 following 6 hours of treatment with levels decreasing thereafter. Data are mean ± SEM and represent an average of four independent experiments/time point. *Significant difference compared to untreated control, $P \leq 0.05$. (**B**) Representative images demonstrating an increase in hBD2 and LL-37 protein expression following 24 hours of treatment with heat-killed *F. solani* (FS) or *C. albicans* (CA; as positive control) compared to untreated control (C). The data are representative of three separate experiments. (**C**) Densitometry of the immunoblots confirmed upregualtion of hBD2 and LL37 protein following *F. solani* and *C. albicans* treatment. Data are mean ± SEM and represent an average of three independent experiments.

ments performed using increasing concentrations of PAMPs or the combination did not show a prominent concentration-dependent effect (data not shown). Therefore in all subsequent experiments cells were treated with 10 μg/ml of PAMPs or the combination. Interestingly, while we did not observe an effect of *F. solani* challenge on hBD1 expression (Fig. 1), 6 and 12 hours of treatment with Zymosan Depleted resulted in an 82.8 ± 39.4– and 34.0 ± 5.3–fold ($P < 0.05$) increase, respectively, while the combination showed a 289.9 ± 2.0– and 49.6 ± 12.1–fold ($P < 0.001$, 0.02 respectively) increase in hBD1 compared to control (Fig. 2A). hBD1 expression also was enhanced by Zymosan challenge alone at 6 and 12 hours but this did not reach statistical significance. hBD2 expression (Fig. 2B) was significantly increased 6 hours after treatment with Zymosan, Zymosan Depleted, and the combination of the two: 50.0 ± 9.9, 37.1 ± 9.8, 450.1 ± 94.1 ($P < 0.05$, 0.05, and 0.001 respectively). At 12 hours after Zymosan treatment levels of hBD2 had returned to baseline, whereas those in Zymosan Depleted and PAMP combination-treated cells remained elevated. hBD3 (Fig. 2C) expression was statistically significantly increased by Zymosan, Zymosan Depleted, and the combination at 6 hours by 49.5 ± 2.7, 248.6 ± 72.1, and 228.3 ± 35.8 ($P < 0.02$, 0.001, 0.001) but by 12 hours only levels in cells treated with the combination remained significantly elevated (100.7 ± 11.6–fold), although less than at 6 hours and these returned to baseline by 24 hours. In keeping with the trends in defensin expression changes, LL37 (Fig. 2D) peaked at 6 hours and was trending toward baseline at 24 hours. Zymosan, Zymosan Depleted, and the combination of the two resulted in a 78.2 ± 2.7–, 183.4 ± 0.97–, and 408.9 ± 107.3–fold increase compared to untreated control ($P < 0.001$, $P < 0.0001$, $P < 0.001$), respectively. Only the combination of Zymosan and Zymosan Depleted showed a statistically significant 99.0 ± 3.3–fold increase at 12 hours ($P < 0.01$).

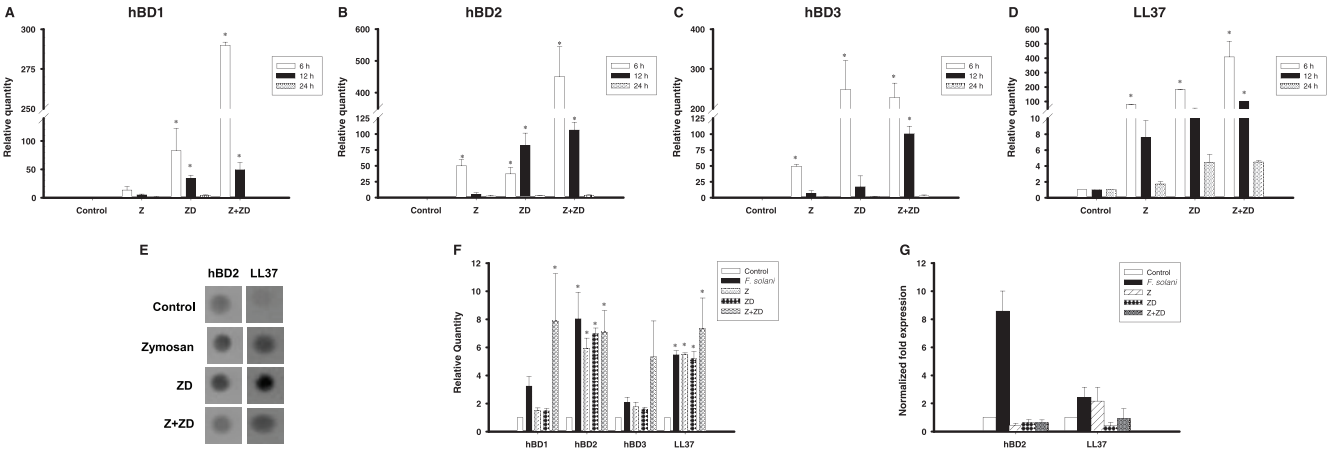

Figure 2. HCEC AMP mRNA Expression is upregulated by Zymosan (Z), Zymosan Depleted (ZD) alone, and in combination (Z+ZD). hTCEpi were exposed to Z, ZD, Z+ZD for 6, 12, and 24 hours, then hBD-1 (**A**), hBD-2 (**B**), hBD-3 (**C**), and LL37 (**D**) expression was determined by RT-PCR. Peak increases in expression were typically at 6 hours with levels trending toward baseline at subsequent time points. Data are mean ± SEM and represent an average of three to five independent experiments. *Significant difference compared to untreated cells, $P \leq 0.05$. (**E**) Increased secretion of hBD2 and LL37 in to the culture media also was detected 24 hours after challenge with Z, ZD, Z+ZD. Data are representative of three independent experiments. (**F**) Primary human corneal epithelial cells treated with heat-killed *F. solani*, and Z, ZD, Z+ZD demonstrated an increase in hBD2 and LL37 expression compared to untreated cells following 6 hours of treatment. Data are mean ± SEM and represent an average of three individual experiments. *Significant difference, $P \leq 0.05$. (**G**) Quantification of hBD2 and LL37 protein in primary HCECs by immunoblot showed an increase in *F. solani*-treated cells but no significant increase in PAMP-treated cells. Data are mean ± SEM and represent an average of three individual experiments.

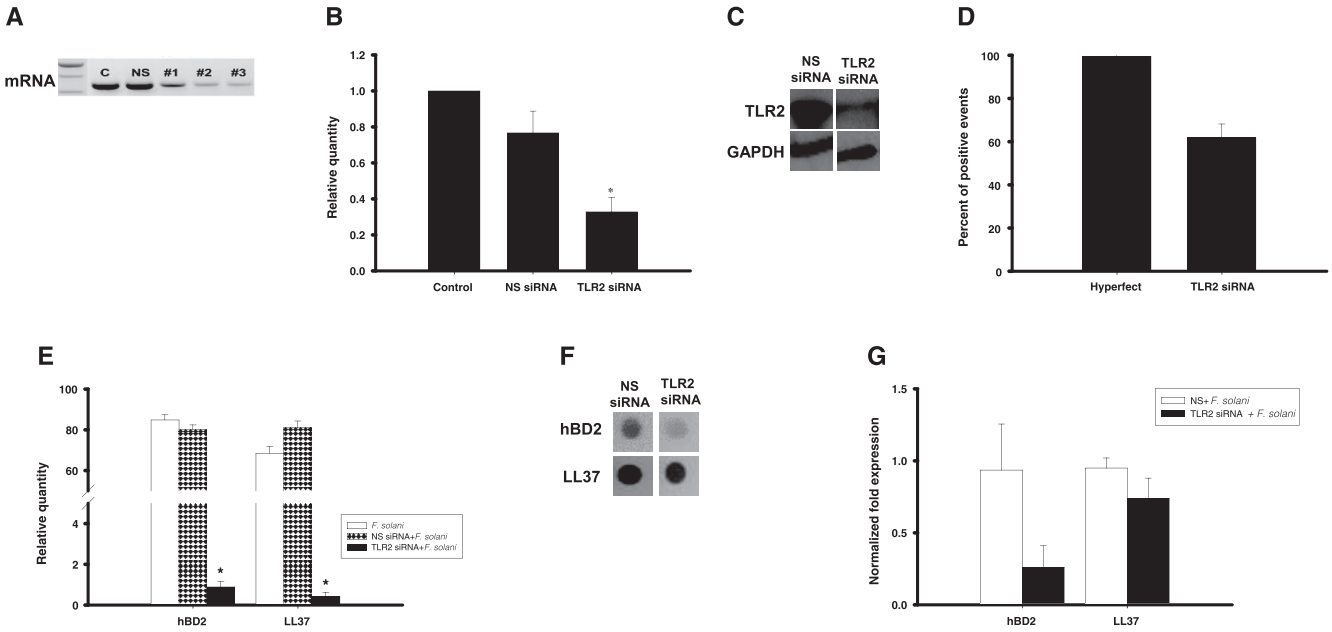

Figure 3. Modulation of AMP expression by fungal activation of TLR2 in hTCEpi cells. (**A**) One-step RT-PCR was used to determine TLR2 knockdown by a series of siRNAs. NS, nonspecific siRNA; #1, #2, and #3, three different TLR2 specific siRNAs. Data are representative of three independent experiments with comparable results. (**B**) Confirmation via real-time relative RT-PCR indicated a significant decrease in TLR2 mRNA expression in siRNA #3–treated cells compared to the NS-treated cells. Data are expressed as relative quantity compared to control ± SEM from 3 separate experiments. *Significant difference ($P < 0.01$) compared to untreated control. (**C**) Western blotting also showed a marked decrease in TLR2 expression following 48 hours of treatment with TLR2 siRNA #3 compared to NS siRNA. GAPDH was used as a loading control as indicated. The data are representative of four independent experiments. (**D**) Flow cytometry in TLR2 siRNA– and NS siRNA–treated cells demonstrated a decrease in TLR2 expression in cells treated with the specific siRNA. Data are representative of three independent experiments. (**E**) siRNA #3 was used to knockdown TLR2 then cells were stimulated with heat-inactivated *F. solani* for 6 hours and AMP mRNA expression determined by RT-PCR. The expression of hBD2 and LL37 mRNA stimulated by *F. solani* was completely abrogated by TLR2 knockdown. Data are expressed as relative quantity change compared to control ± SEM from 5 separate experiments. *Significant difference compared to NS siRNA. (**F**) siRNA #3 was used to knockdown TLR2 then cells were stimulated with heat-inactivated *F. solani* for 24 hours. Immunoblotting then was performed and showed a decrease in hBD2 and LL37 secretion in TLR2 siRNA-treated cells. These data are representative of three or four independent experiments with comparable findings. NS, nonspecific siRNA+ *F. solani*; TLR2, TLR2 siRNA+ *F. solani*. (**G**) Quantification of the hBD2 and LL37 immunoblots by densitometry. Data are mean ± SEM and represent an average of three individual experiments.

Overall peak upregulation of AMP mRNA was typically at 6 hours after which time levels began to fall and were almost back to baseline by 24 hours. At 6 hours after treatment AMP mRNA upregulation was significantly greater with the combination of PAMPs compared to either Zymosan or Zymosan Depleted challenge alone ($P < 0.001$) with the exception that for hBD3 this applied only to the comparison of the PAMP combination to Zymosan alone ($P < 0.02$). Cells treated with the PAMPs for 24 hours also showed an increase in hBD2 and LL37 protein secretion compared to nontreated cells (Fig. 2E). Quantitation (not shown) of the secreted hBD2 and LL37 in hTCEpi cells was consistent with the representative immunoblot images. To confirm that similar effects also were seen in primary cultured HCEC, AMP expression was determined following 6 hours of treatment with heat-killed *F. solani*, Zymosan, Zymosan Depleted, or the combination of the two. As shown in Figure 2F there was upregulation of all AMPs tested. Statistically significant upregulation was observed for hBD2 and LL37 with all of the treatments. Increases of 8.0 ± 1.9- and 5.5 ± 0.2-fold compared to control ($P < 0.008$, 0.004) were observed following treatment with heat-inactivated *F. solani* for hBD2 and LL37 respectively. Treatment with Zymosan resulted in an upregulation of hBD2 and LL37 by 5.9 ± 0.7- and 5.5 ± 0.1-fold, Zymosan Depleted by 6.9 ± 0.4- and 5.2 ± 0.5-fold, and the combination by 7.1 ± 1.5- and 7.3 ± 2.2-fold compared to untreated control ($P < 0.03$, 0.04, 0.004). Although expression of hBD-1 and -3 was increased, this only reached statistical significance for hBD1 in cells exposed to the combination of Zymosan and Zymosan Depleted. AMP secretion was determined by immunoblotting following 24 hours of incubation with *F. solani* and PAMPs and showed similar results to those of hTCEpi cells although the intensity of staining was much lower, which is in keeping with the lower levels of AMP mRNA expression observed in primary cultured cells compared to the cell line. Relative densitometry of AMP protein levels in cell supernatant of primary HCECs is shown in Figure 2G. Data confirmed an increase in hBD2 and LL37 by 8.58 ± 1.4- and 2.45 ± 0.7-fold in *F. solani*-treated cells respectively, although there was minimal detectable protein upregulation in response to PAMPs.

Modulation of AMP Expression by TLR2

As AMP expression is known to be modulated by TLR activation and previous studies have suggested that TLR2 is an important fungal PRR, we sought to determine if it is involved in *F. solani*-induced AMP expression in hTCEpi cells by knocking down its expression by siRNA. As shown in Figure 3A, 3 different siRNA's #1, 2, and 3 (s167, s168, and s170) were tested and resulted in a 45%, 67%, and 72% decrease, respectively, in TLR2 mRNA expression compared to control. The effectiveness of knockdown by siRNA #3 (s170) then was confirmed by relative quantitative RT-PCR as shown in Figure 3B, where a 76% ± 12% decrease in TLR2 mRNA levels was found. Reduction in TLR2 protein expression when cells were transfected with siRNA #3 (s170) was confirmed by Western blotting (Fig. 3C) and flow

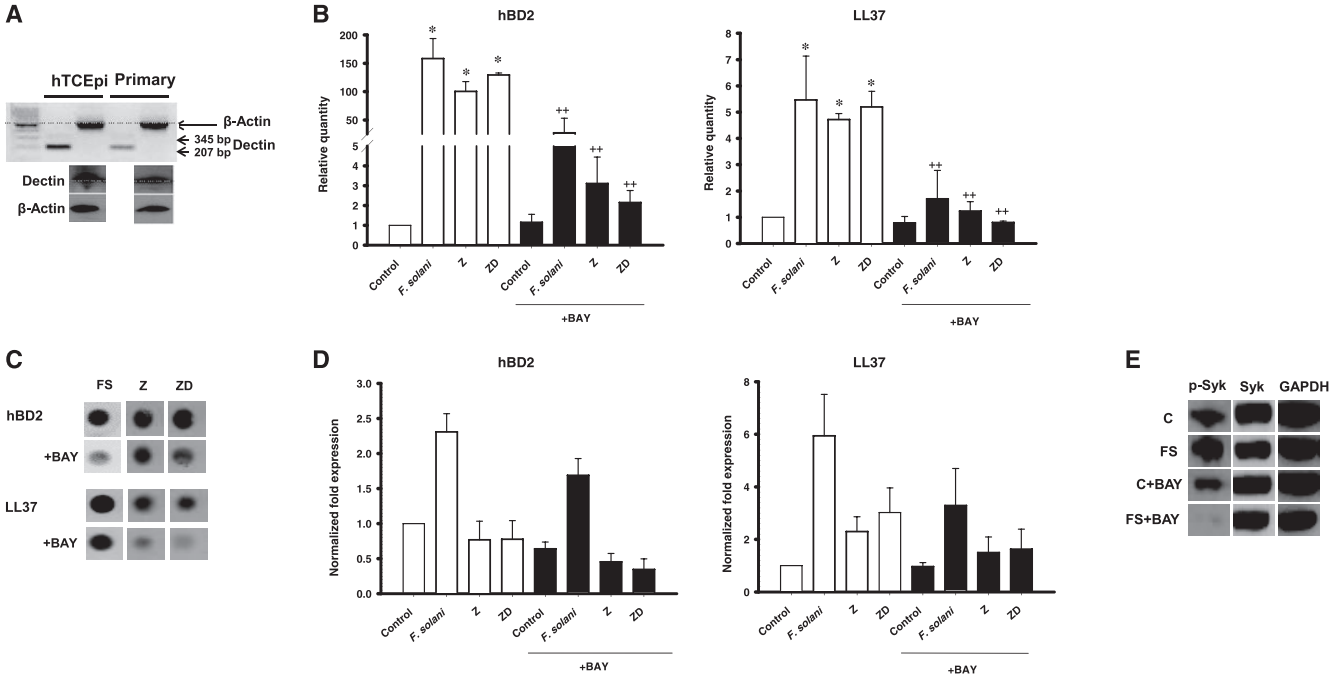

Figure 4. BAY 61-3601, a Syk pathway inhibitor, attenuates fungal and PAMP-stimulated AMP expression by hTCEpi cells. (**A**) One-step RT-PCR and Western blotting showed that primary cultured HCEC and hTCEpi cells had robust expression of Dectin-1 (major isoform 207 bp, minor 345 bp) at the mRNA (*upper*) and protein level (*lower*). Data are representative images from three independent experiments. hTCEpi cells were treated with Syk inhibitor BAY 61-3601 for 48 hours then stimulated with heat-killed *F. solani* or PAMPs for 6 hours (mRNA) or 24 hours (protein) and AMP expression determined by RT-PCR (**B**) and immunoblotting (**C**). Pretreatment with BAY 61-3601 (BAY) reduced *F. solani* (FS)/PAMP-induced AMP expression. In (**B**) data are expressed as relative quantity change compared to control ± SEM from 3 separate experiments. *Upregulation of hBD2 and LL37 compared to untreated or BAY treated control. ++, Significant downregulation following BAY treatment compared to their respective controls. Immunoblot data (**C**) are representative of two and three independent experiments for hBD2 and LL37, respectively. (**D**) Relative densitometry data for hBD2 and LL37 protein in *F. solani*- and PAMP-treated cells. (**E**) Western blotting for total and p-Syk demonstrated an increased in p-Syk in *F. solani*–treated cells compared to control (C) which was abrogated by treatment with BAY. GAPDH was used as the loading control. Data are representative of three independent experiments.

cytometry (Fig 3D). This siRNA#3 (s170) then was used in subsequent experiments. The effects of TLR2 knockdown on AMP expression are shown in Figures 3E and 3F. Figure 3D demonstrates flow cytometry data indicating a knockdown of TLR2 in siRNA treated hTCEpi cells compared to nonspecific siRNA-treated cells. As seen previously (Fig. 1), exposure to heat-inactivated *F. solani* stimulated hBD2 and LL37 mRNA expression (by 84.9– and 68.5–fold respectively, $P < 0.0001$, 0.0001) and in these experiments this was completely abrogated ($P < 0.0001$, 0.0001) in cells in which TLR2 had been knocked down by siRNA (Fig. 3E). Nonspecific siRNA did not significantly interfere with the ability of heat-inactivated *F. solani* to stimulate AMP expression. Figure 3F (immunoblotting) and quantitation by densitometry (Fig. 3G) confirms reduction of *F. solani*-stimulated hBD2 and LL37 secretion in to the culture media in cells treated with the TLR2-specific siRNA versus the nonspecific siRNA.

Dectin-1 Mediated Modulation of AMP Expression Using Syk Inhibitor

First the presence of Dectin-1 was confirmed in the hTCEpi cell line using primary cultured HCEC as a positive control.[23] As shown in Figure 4A, primary cultured HCEC and hTCEpi cells expressed mRNA for the 2 isoforms of Dectin-1 (major 207 and minor 345 base pairs [bp]) and Dectin-1 protein expression was detectable in cell lysates. To determine the role of Dectin-1 in *F. solani*-induced AMP expression, we used BAY 61-3601 to block Syk signaling. Quantitation of hBD2 and LL37 mRNA as demonstrated in Figure 4B indicated that exposure to *F. solani* or Zymosan or Zymosan Depleted showed a significant upregulation of hBD2 and LL37 as we had seen previously. However, pretreatment of cells with BAY 61-3601 followed by stimulation with *F. solani*, Zymosan, or Zymosan Depleted resulted in significantly decreased expression of hBD2 by 4.8-, 31.2-, and 59.2–fold ($P < 0.001$, 0.01, and 0.001) and LL37 by 2.2-, 2.8-, and 5.5-fold ($P < 0.04$, 0.04, and 0.01), respectively. Data in Figure 4C show that exposure to BAY 61-3601 followed by treatment with heat-killed *F. solani*, Zymosan, or Zymosan Depleted results in reduced secretion of hBD2 and LL37 compared to their respective controls. hBD2 secretion induced by heat-killed *F. solani*, Zymosan, and Zymosan Depleted was reduced by 26.9% ± 0.2%, 55.0% ± 11.4%, and 43.8% ± 15.8%, respectively. Similarly, there was a decrease in LL37 secretion in BAY 61-3601 pretreated heat-killed *F. solani*, Zymosan, and Zymosan Depleted treated samples of 35.0% ± 7.6%, 46.2% ± 2.4%, and 50.7% ± 4.4%, respectively (mean ± SD). Figure 4D shows quantitative densitometry data for hBD2 and LL37 protein expression in *F. solani*- and PAMP-treated cells. These data demonstrated that exposure to BAY 61-3601 decreased hBD2 and LL37 expression compared to stimulated controls, although due to variability this did not reach statistical significance. Figure 4E shows upregulation of p-Syk in *F. solani*-treated cells compared to control. Pretreatment with BAY 61-3601 decreased the expression of p-Syk stimulated by *F. solani*, indicating a critical role played by the Syk pathway in AMP upregulation following fungal treatment.

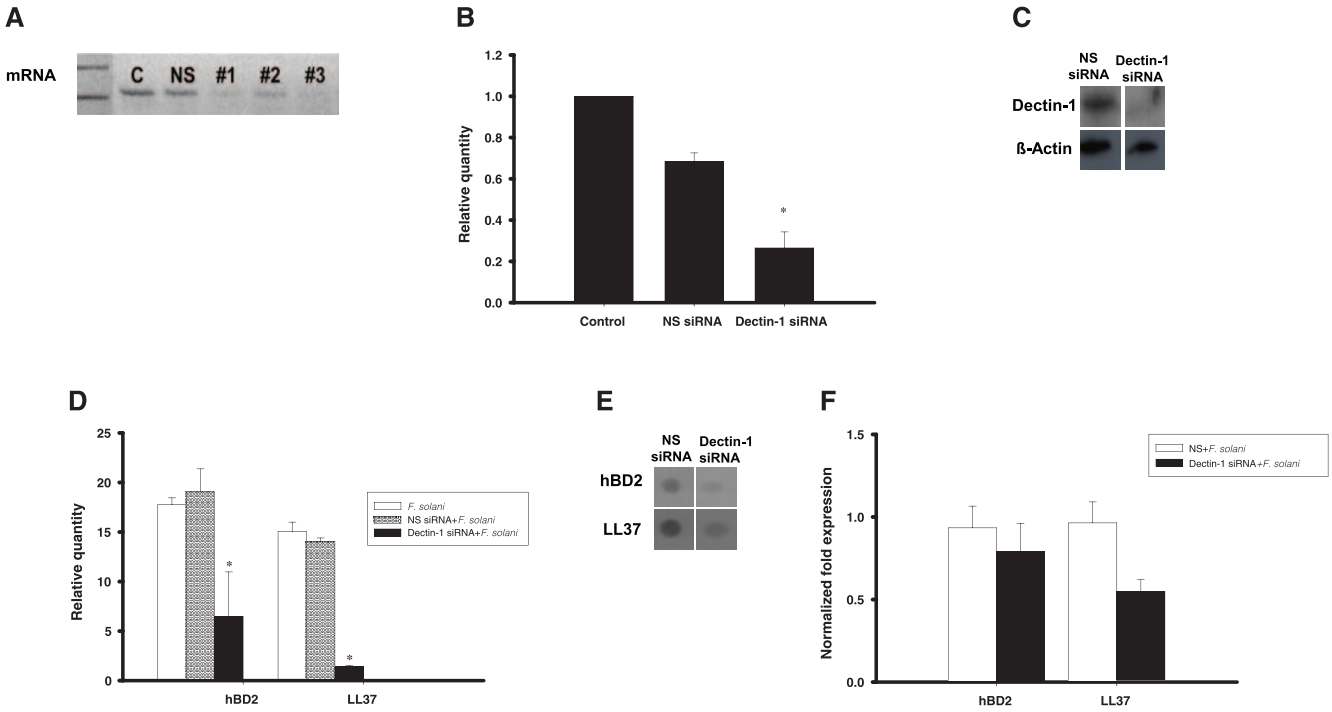

Figure 5. Dectin-1 knockdown attenuates fungal stimulated AMP expression by hTCEpi cells. (**A**) One step RT-PCR was used to determine Dectin-1 knockdown by a series of siRNAs after 48h. All of the siRNAs were effective and #3 (s227204) was selected for further study. The data are representative of three independent experiments. (**B**) Relative quantitative RT-PCR confirmed efficient knockdown of Dectin-1 by siRNA s227204. Data are expressed as relative quantity change compared to control ± SEM from 4 separate experiments. (**C**) Western blotting also showed a decrease in Dectin-1 protein expression compared to NS siRNA with β-Actin as the loading control. The data are representative of two independent experiments. (**D**) Following knockdown of Dectin-1 the cells were stimulated with heat-inactivated *F. solani* and AMP mRNA expression determined by RT-PCR. The expression of hBD2 and LL37 mRNA stimulated by *F. solani* was significantly decreased compared to NS siRNA-treated cells. Data are expressed as relative quantity change compared to control ± SEM from 5 separate experiments. *Significant difference compared to nonspecific siRNA. (**E**) Immunoblotting for AMPs, hBD2, and LL37 in *F. solani* stimulated cells following Dectin-1 or nonspecific siRNA treatment. Dectin-1 siRNA knockdown resulted in decrease in hBD2 and LL37 secretion. These data are representative of three to four independent experiments with comparable findings. (**F**) Shows relative densitometry data for the immunoblots showing reduced hBD2 and LL37 expression.

Modulation of AMP Expression by Knocking Down Dectin-1 Receptor

To confirm the specific involvement of Dectin-1 suggested by our experiments inhibiting the Syk pathway, siRNA was used to knockdown expression of Dectin-1 and AMP expression in *F. solani*-treated hTCEpi cells determined. As depicted in Figure 5A, hTCEpi cells were treated with Dectin-1 siRNAs or nonspecific siRNA. The expression of Dectin-1 was significantly knocked down by 70.2% ± 5.8%, 67.6% ± 2.8%, and 77.8% ± 3.1% by the three siRNAs (#1-s34783, #2-s34785, and #3-s227204), respectively. Further, confirmation of the knockdown by siRNA #3- s227204 as evaluated by relative quantitative RT-PCR demonstrated a 75.9% ± 7.8% knockdown as shown in Figure 5B. Therefore, siRNA #3-s227204 was used in subsequent experiments. Figure 5C confirms the decrease in Dectin-1 expression at the protein level following treatment with Dectin-1 siRNA compared to nonspecific siRNA. As demonstrated in Figure 5D heat-inactivated *F. solani* and nonspecific siRNA treatment before *F. solani* stimulated expression of hBD2 and LL37 as seen previously. Silencing Dectin-1 significantly decreased the *F. solani* stimulated expression of hBD2 from 17.4 ± 0.7 to 6.4 ± 4.4 and of LL37 from 15.0 ± 0.9 to 1.4 ± 0.2 at the mRNA level ($P < 0.04$ and 0.0001). Representative immunoblots and densitometry analysis for hBD2 and LL37 in Dectin-1 siRNA– and nonspecific siRNA–treated cells are shown in Figures 5E and 5F, respectively. The data demonstrated that Dectin-1 knockdown decreased hBD2 and LL37 expression compared to nonspecific siRNA-treated control.

Functional Significance of *F. solani* Induced hBD2 and LL37 Secretion

To determine if the observed upregulation and enhanced secretion of hBD2 and LL37 was of functional significance, supernatants from hTCEpi cells treated with *F. solani*, Zymosan, or Zymosan Depleted were incubated with live *F. solani* and the percent killing by the culture supernatants was determined. As shown in Figure 6A supernatants from heat-killed *F. solani*, Zymosan, and Zymosan Depleted treated cells showed significantly increased killing of *F. solani* by 58.4% ± 4.0%, 82.7% ± 6.0%, and 90.5% ± 4.2%, respectively ($P <$ 0.0001) compared to control (culture media from untreated cells). We then performed additional experiments in the presence of antibodies to block hBD2 and LL37 antimicrobial activity. Figure 6B shows that blocking hBD2 activity in supernatant from fungal or PAMP-stimulated cells decreased the percent killing of *F. solani* by 83.2% ± 6.5%, 67.1% ± 5.6%, and 82.1% ± 4.8% ($P < 0.002$, 0.01, 0.002) compared to the respective IgG controls. Similarly, as shown in Figure 6C, the presence of LL37 antibody decreased percent killing by 99.3% ± 5.9%, 98.9% ± 3.3%, and 84.7% ± 5.8% ($P < 0.0001$, 0.0001, 0.001) compared to the serum control.

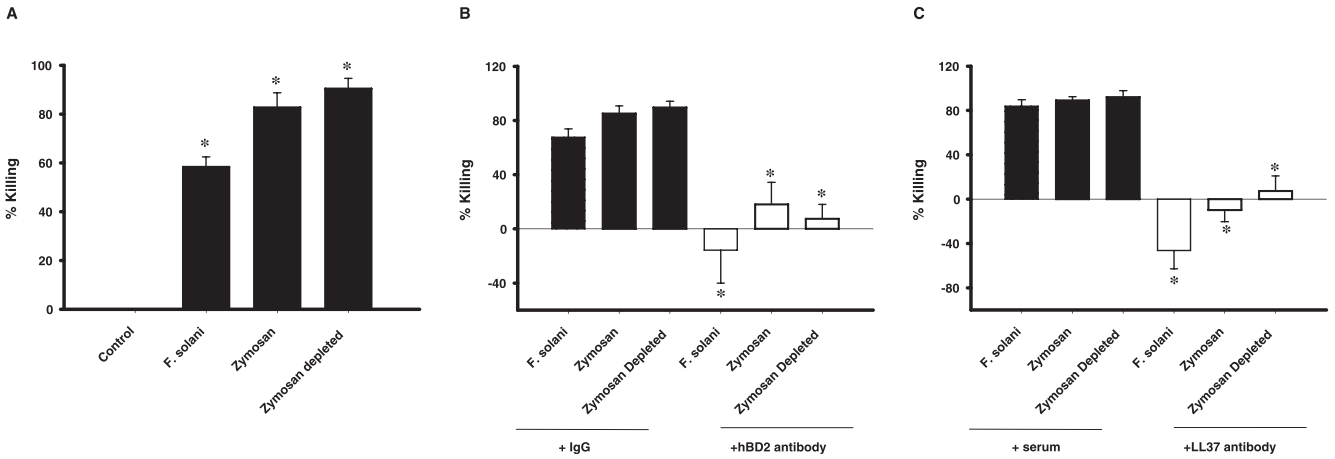

Figure 6. Functional significance of *F. solani* induced AMP upregulation. (**A**) Antifungal assays were performed using culture supernatants from hTCEpi cells treated with heat-killed *F. solani* or PAMPs for 24 hours. *Significant increase in percent killing compared to control. Specific antibodies to hBD2 (**B**) and LL37 (**C**) were used to block the antimicrobial activities of these AMPs, under which circumstances the killing activity of the culture supernatants was lost. *Significant decrease in percent killing compared to the respective control. All data are mean ± SEM from three to four separate experiments.

Discussion

F. solani is a major cause of mycotic keratitis resulting in visual impairment and blindness worldwide; however, the intricate mechanisms of the host-immune response to this pathogen invading the cornea are not well established. Although recent studies have addressed the role of various PRRs, including TLRs and Dectin-1 in fungal keratitis, the precise mechanism involved still remains elusive.[27,34–37] Collectively, results from this investigation demonstrated that Dectin-1 and TLR2 are vital PRRs expressed in HCEC. Our findings showed that PAMPs associated with *F. solani* upregulate AMPs hBD2 and LL37 at the mRNA and protein level via a TLR2- and Dectin-1–mediated mechanism. Furthermore, our data also revealed that hBD2 and LL37 have functional significance as they mediate the antifungal activity of culture supernatants from *F. solani* and PRR agonist–treated HCEC.

A number of previous studies have shown upregulation of AMPs in response to fungal exposure in several cell types, including HCEC in which hBD-2, -3, and LL37 were upregulated in response to *C. albicans* and *A. fumigatus* in vitro.[31,38] The cathelicidin CRAMP also was reported to be upregulated in a murine model of *Candida* keratitis.[39] We have shown that several β-defensins and CRAMP are elevated in a murine model of *Fusarium* keratitis,[15] but there have been no comprehensive studies investigating the complement of AMP expression in HCEC in response to *F. solani*. We found exposure to this fungal pathogen significantly and reproducibly increased expression of hBD2 and LL37 in a human corneal epithelial cell line and we confirmed this in primary cultured cells. AMP mRNA levels peaked at 6 hours, then began to decline but elevated AMPs were detected in the culture media 24 hours later. We also showed that fungal glucan (in the form of Zymosan or Zymosan Depleted) initiated the enhanced AMP expression. HCEC expression of two defensins, hBD1 and hBD3, was not significantly upregulated by *F. solani* but marked increases were apparent when the cells were challenged with PAMPS alone or in combination. These findings may be because of the presence of highly concentrated PAMPs in the purified compounds compared to heat-inactivated *F. solani*. Interestingly, the combination of Zymosan and Zymosan Depleted demonstrated a significant upregulation of AMPs tested compared to individual PAMP treatment after 6 hours. Generally, this was more than a simple additive effect indicating cooperation among TLR2 and Dectin-1, the two primary targets for these PAMPs. We also observed that while the trends of AMP upregulation were similar between the hTCEpi cell line and primary cultured cells, the relative fold changes seen in the primary cultured cells were much lower compared to the cell line. As far as possible, we confirmed our mRNA expression findings at the protein level and quantitated the immunoblots by densitometry. However as AMPs are challenging to detect owing to their small size, relatively low expression and lack of optimal antibodies, these data, in contrast to the mRNA, often did not show statistically significant changes, although the trends were the same.

As previous studies have reported the involvement of TLRs, particularly TLR-2 and -4 and Dectin-1 in fungal effects caused by *A. fumigatus*, *C. albicans, F. solani*,[27,33,40–42] we sought to address the role of these PRRs in mediating the upregulation of AMPs in *F. solani* challenged HCEC. We focused on involvement of TLR2 and Dectin-1, as expression of functional TLR4 and its necessary accessory molecules in HCEC have been questioned and studies with our cultured cells failed to show a robust response to the classic TLR4 ligand lipopolysaccharide (data not shown).[43,44] Data from our investigation showed that hTCEpi cells and primary cultured HCEC expressed PRRs Dectin-1 and TLR2 at mRNA and protein levels as demonstrated by PCR, and Western blot. Human corneal epithelial cell TLR2 expression has been documented in a number of studies[16] but few studies have addressed Dectin-1 expression in cultured HCECs. Hua et al.[24] showed that Dectin-1 was expressed in the epithelium of human corneal tissue sections and in primary cultured human corneal limbal epithelial cells.[24] Interestingly, their study also showed upregulation of Dectin-1 following exposure to heat-killed *C. albicans*. Another recent study reported the expression of Dectin-1 in an unspecified corneal epithelial cell line and in corneas from patients with fungal keratitis.[23] Our data confirm the expression of Dectin-1 in primary cultured HCEC and reveals its expression by a telomerase modified cell line. We also observed Dectin-1 expression (data not shown) in SV40 immortalized HCECs.[45]

Dectin-1 signaling involves Syk or CARD as reported by Poeck et al.[46] To study the involvement of Dectin-1 in AMP modulation, a potent pharmacologic Syk inhibitor BAY 61-3606 was added before agonist treatment.[47] Under these conditions, AMP expression was significantly decreased indicating that AMP modulation occurs via a Syk-mediated mechanism. We also used

siRNA to knockdown Dectin-1 expression and confirmed that it is necessary for *F. solani*-stimulated AMP upregulation in HCEC. Here, we also have shown that blocking TLR2 expression using specific siRNA significantly decreased the *F. solani*-mediated increase in expression of hBD2 and LL37. Thus, Dectin-1 and TLR2 can mediate the effects of *F. solani* on AMP expression in HCEC. Although we were not able to study involvement of TLR4 in our specific cells, based on observations in other studies[27,42] this PRR also is likely to be involved in the ocular surface epithelial response to *F. solani* in vivo.

Having shown that *F. solani* and PAMPs stimulated HCEC secretion of AMPs hBD2 and LL37, we also studied the functional significance of this via antifungal assays. We observed that blocking the activity of hBD2 or LL37 using specific antibodies significantly decreased the antifungal activity of the cell culture supernatant collected from *F. solani*-treated cells. Interestingly, addition of either AMP antibody was able to completely block the antifungal activity, suggesting that hBD2 and LL37 in the culture media cooperate to achieve effective killing of *F. solani*. This is in keeping with many previous studies, which have suggested that AMP antimicrobial activity is due in large part to cooperative interactions among two, or possibly more AMPs.

Overall our data suggested that AMPs, hBD2 and LL37 in particular, upregulated via TLR2 and Dectin-1 in response to *F. solani*, have functional antifungal activity that will help facilitate eradication of fungal pathogens and that corneal epithelial cells are active participants in the ocular surface innate defense against fungal keratitis.

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